SPECIFICATION

CROSSLINKED POLYSACCHARIDE MICROPARTICLES AND
METHOD FOR THEIR PREPARATION

TECHNICAL FIELD

5   [0001]   The present invention relates to sustained-release drug carriers composed of crosslinked polysaccharide microparticles which allow sustained release of drugs, more particularly pharmacologically active proteins or peptides, as well as a method for their

10 preparation.

BACKGROUND ART

[0002]   In recent years, an increasing number of formulations of pharmacologically active proteins or peptides have been developed for commercial use. However,

15 such drugs usually have a short half-life in the blood and most of them are injections that must be administered repeatedly at frequent intervals, thus imposing excessive burdens on patients during drug administration. Hence, there is a demand for practical, sustained-release

20 formulations of protein or peptide drugs, which exert their efficacy in as small amounts as possible and which permit reduced frequency of administration.

[0003]   Sustained-release formulations of pharmacologically active proteins or peptides will cause

25 denaturation or aggregation of the proteins or peptides during formulation preparation or sustained release, which results in a reduced recovery rate and constitutes a major obstacle to their development for commercial use. It has

been attempted to prepare sustained-release formulations

based on a biodegradable polymer matrix such as polylactic

acid-polyglycolic acid copolymer (PLGA), but such

formulations have been reported to cause protein

5   denaturation and/or aggregation due to the hydrophobicity

of the matrix, a drying step and/or a decrease in pH (see

Non-patent Documents 1 and 2). On the other hand, there

are also reports of sustained-release formulations based on

a hydrophilic hydrogel matrix with reduced risks of these

10   problems, but such formulations are not ready for

commercial use. In terms of safety, a material used as a

sustained-release matrix should combine non-antigenicity,

non-mutagenicity, non-toxicity and biodegradability. Thus,

no sustained-release formulation is now ready for

15   commercial use in all aspects, i.e., encapsulation

efficiency and recovery rate of proteins or peptides, as

well as safety.

[0004]   Some recent reports have proposed the use of

polysaccharides as matrixes for drug carriers. Among them,

20   hyaluronic acid (HA), a biomaterial (polysaccharide)

isolated from the vitreous body of bovine eyes in 1934 by K.

Meyer, has been known as a major component of extracellular

matrix for a long time. HA is a kind of glycosaminoglycan

composed of disaccharide units in which D-glucuronic acid

25   and N-acetylglucosamine are linked to one another via

$\beta(1\rightarrow3)$glycosidic linkages.

[0005]   There is no difference among species in the

chemical and physical structure of HA and humans also have

a metabolic system for HA; HA is therefore one of the safest medical biomaterials in terms of immunity and toxicity. Recent years have enabled microbial mass production of high-molecular-weight HA and also have allowed commercial use of HA in the fields of therapeutic agents for degenerated cartilage, cosmetics, etc.

[0006] There are also many reports of crosslinking techniques for HA as a matrix and sustained release formulations of protein or peptide drugs from HA gels. Techniques known for gelling HA via chemical crosslinking include the carbodiimide method (see Patent Document 1), the divinylsulfone method (see Patent Document 2), and the glycidyl ether method (see Patent Document 3). In general, when a protein or peptide is introduced into a crosslinked gel for encapsulation purposes, it results in a low introduction efficiency because of problems arising from compatibility and electrostatic repulsion between HA and the protein or peptide. In contrast, when *in situ* crosslinking is performed in the presence of a protein or peptide, it is advantageous in that the protein or peptide can be held in a gel at a high encapsulation efficiency. There are some reports showing that such *in situ* crosslinking is adapted for encapsulation of proteins or peptides into HA gels to give sustained-release formulations (see, e.g., Patent Document 4). However, there arises a problem of recovery rate when such an approach is used for *in situ* crosslinking of HA in the presence of proteins or peptides to prepare sustained-

release formulations. As an example, a method is reported in which a HA derivative (HA-HZ) modified to have a hydrazide group (HZ) is crosslinked with a crosslinking agent comprising N-hydroxysuccinimide (NHS) (see Patent

5   Document 5). This method is intended for *in situ* crosslinking under physiological conditions and limits crosslinkage formation at pH 7.4 to pH 8.5. However, the inventors' investigations have confirmed that this method also results in low recovery rates of proteins or peptides

10   from the thus obtained HA gel. This is because the proteins or peptides will be partially reacted (mainly at their amino groups) with the crosslinking agent during crosslinking reaction to give crosslinked proteins. This method also suffers from a problem in that denatured

15   proteins or peptides remaining in the gel have reduced biological activity and, if anything, are responsible for the cause of antigenicity. Although it is an essential requirement for pharmaceutical preparations that the encapsulated drug is released at a high recovery rate, no

20   method is known for chemically crosslinking and gelling HA without causing proteins or peptides to react. Also, another method has been reported to encapsulate proteins or peptides at high recovery rates, in which polyethylene glycol (PEG) is used as a matrix and crosslinked through

25   nucleophilic addition reaction of unsaturated functional groups (see Patent Document 6), but this method suffers from a problem in that fragments of non-biodegradable PEG remain unabsorbed.

[0007]   In actual fact, to formulate such sustained-release materials into injectable formulations, these materials should be formulated in the form of microparticles. Spray dryers are widely used in such attempts and are also reported to be used in formulating insulin (see Non-patent Documents 3 and 4) and rh anti-IgE antibody (see Non-patent Document 5) in the form of microparticles, as well as in encapsulated drugs into hyaluronic acid microparticles (see Patent Documents 7 and 8). However, since the microparticles thus obtained will be dissolved in a short time in the subcutaneous tissues, they have a very short period of sustained release and are less practical for sustained release purposes. There is another report in which chitosan is crosslinked during spray drying so as to encapsulate low-molecular drugs therein (see Non-patent Document 6). However, the release period is as short as a few minutes in this case, and aldehyde used as a crosslinking agent is highly reactive with a functional group such as an amino group and hence cannot be used for proteins, peptides and other low-molecular drugs having a functional group(s) such as an amino group.

Patent Document 1: International Publication No. WO94/02517
Patent Document 2: JP 61-138601 A
Patent Document 3: JP 5-140201 A
Patent Document 4: United States Patent No. 5827937
Patent Document 5: International Publication No. WO95/15168
Patent Document 6: International Publication No. WO00/44808

Patent Document 7: Japanese Patent No. 3445283

Patent Document 8: International Publication No. WO96/18647

Non-patent Document 1: J. Pharm. Sci., vol. 88, pp. 166-173, 1999

Non-patent Document 2: J. Microencapsulation, vol. 15, pp. 699-713, 1998

Non-patent Document 3: Int. J. Pharm. 233, 227-237, 2002

Non-patent Document 4: J. Control. Rel. 91, 385-394, 2003

Non-patent Document 5: Biotech. and Bioeng. 60, 301-309, 1998

Non-patent Document 6: Int. J. Pharm. 187, 53-65, 1999

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0008]   As stated above, no method is known for preparation of injectable and biodegradable gel microparticles, which satisfies a high encapsulation efficiency, a high recovery rate and safety through *in situ* chemical crosslinking, drying, microparticle formation and drug encapsulation while retaining the biological activity of drugs such as proteins or peptides. Moreover, no example is known for long-acting sustained-release formulations of drugs such as proteins or peptides, which are prepared using such a method.

MEANS FOR SOLVING THE PROBLEMS

[0009]   As a result of extensive and intensive efforts made to overcome the problems stated above, the inventors of the present invention have found that drugs such as proteins or peptides can be efficiently encapsulated while

retaining their biological activity when a solution of a drug and a hyaluronic acid derivative having a crosslinkable functional group(s) is concentrated starting from a dilute state, where crosslinking proceeds slowly, to

5   reach a concentration which facilitates crosslinking, to thereby cause crosslinking reaction between HA molecules during concentration, so that the drug is encapsulated into the crosslinked hyaluronic acid. The inventors have also found that the crosslinked hyaluronic acid microparticles

10   thus obtained are injectable and most suitable as biodegradable and safe microparticle carriers for long-term sustained release for the purpose of encapsulating drugs such as proteins or peptides. These findings led to the completion of the present invention.

15   [0010]   Namely, the present invention relates to injectable sustained-release formulations of drugs such as proteins or peptides encapsulated in gels, which are obtained through *in situ* crosslinking, microparticle formation and drying while retaining the biological

20   activity of the drugs such as proteins or peptides, as well as a method for their preparation.

[0011]   Namely, in one aspect, the present invention provides a method for preparing crosslinked polysaccharide microparticles, which comprises the following steps:

25   a)   preparing a dilute solution containing a polysaccharide derivative having a crosslinkable functional group(s);

b)   dispersing the solution to form microparticulate

droplets; and

c)   concentrating the solution contained in the droplets to facilitate crosslinking reaction of the polysaccharide derivative.   In another aspect, the present invention further provides such a method wherein step b) is a step in which the solution is dispersed by spraying to form microparticulate droplets.

[0012]   In yet another aspect, the present invention also provides crosslinked polysaccharide microparticles which can be prepared by the above method.

[0013]   The present invention will be further described in more detail below.

[0014]   The polysaccharide derivative used in the present invention is not limited in any way as long as it is a polysaccharide derivative having a crosslinkable functional group(s). Among derivatives of glycosaminoglycans (acidic mucopolysaccharides including hyaluronic acid, chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin, heparan sulfate, keratan sulfate, etc.), preferred are those having a crosslinkable functional group(s), and particularly preferred are hyaluronic acid derivatives having a crosslinkable functional group(s).

[0015]   Thus, in yet another aspect, the present invention also provides a method for preparing crosslinked hyaluronic acid microparticles, which comprises the following steps:

a)   preparing a dilute solution containing a hyaluronic

acid derivative having a crosslinkable functional group(s);

b)   dispersing the solution to form microparticulate droplets; and

c)   concentrating the solution contained in the droplets

5   to facilitate crosslinking reaction of the hyaluronic acid derivative. In another aspect, the present invention further provides such a method wherein step b) is a step in which the solution is dispersed by spraying to form microparticulate droplets. In yet another aspect, the

10   present invention also provides crosslinked hyaluronic acid microparticles which can be prepared by the above method.

[0016]   The dilute solution found in step a) of the present invention is a solution containing substances such as a substrate and a reagent required for crosslinking

15   reaction. However, the dilute solution is highly diluted with a solvent and hence the reaction does not proceed or proceeds very slowly in this solution. Although its concentration is not limited in any way, it is for example 0.1% to 5%, and particularly 0.2% to 3%. The solvent used

20   in the present invention may be any solvent commonly used in the art or a mixture thereof and includes, without any limitation, water, DMSO, ethanol, N-methylpyrrolidone, and supercritical carbon dioxide fluid.

[0017]   In step b) of the present invention, the dilute

25   solution may be dispersed in the form of microparticulate droplets by using any technique commonly used in the art. Examples include, without any limitation, techniques for spraying the dilute solution and techniques to form an

emulsion by mixing the dilute solution with another fluid. The microparticulate droplets thus obtained may have an average particle diameter of, for example, 0.04 μm to 1.5 mm, preferably 0.1 μm to 500 μm, without being

5   particularly limited thereto.

[0018]   In step c) of the present invention, the solution may be concentrated by any means as long as it allows the solution to be concentrated to reach a concentration at which the crosslinking reaction is further

10   facilitated. This concentration step is also intended to include, e.g., a state where the solvent is completely removed such that the crosslinking reaction proceeds as a solid phase reaction.

[0019]   Alternatively, the above steps b) and c) may be

15   carried out in a single process. More specifically, the above steps b) and c) may be carried out in a single process by using techniques such as spray drying method, emulsion solvent evaporation method, and solvent diffusion method. Among them, spray drying is preferred for carrying

20   out the above steps b) and c) in a single process.

[0020]   The crosslinked polysaccharide microparticles of the present invention can be prepared when a solution containing a polysaccharide derivative having a crosslinkable functional group(s) is concentrated starting

25   from a dilute state, where crosslinking proceeds slowly, to reach a concentration which facilitates crosslinking, to thereby crosslink the polysaccharide derivative during concentration. Likewise, the present invention is also

characterized in providing drug-carrying microparticles
that are obtained when a solution containing a drug and a
polysaccharide derivative having a crosslinkable functional
group(s) is concentrated starting from a dilute state,

5   where crosslinking proceeds slowly, to reach a
concentration which facilitates crosslinking, to thereby
cause crosslinking reaction during concentration, in
parallel with drying, so that the drug is encapsulated into
the crosslinked polysaccharide.

10 [0021]   The method provided by the present invention
and the crosslinked polysaccharide microparticles (e.g.,
crosslinked hyaluronic acid microparticles) obtained by the
method preferably have characteristics as shown below.

1.   The method and microparticles ensure complete

15 biodegradability and *in vivo* safety.

2.   By grafting a polysaccharide such as HA with a
crosslinkable functional group(s), the method and
microparticles can keep a very short distance between
crosslinking points (e.g., in the case of HA, about 3 nm

20 when grafted at 33 mol% based on glucuronic acid) and are
advantageous in achieving long-term sustained release.

3.   The method and microparticles achieve a high
crosslinking density.

4.   When a protein is used as a drug, the method and

25 microparticles can prevent protein denaturation.

5.   Microparticle formation, drying and crosslinking can
be carried out in a single process.

[0022]   As used herein, the term "crosslinking" or

"chemical crosslinking" is intended to mean containing intermolecular or intramolecular crosslinkages via covalent bonds. It may also mean having both intermolecular and intramolecular crosslinkages.

5   [0023]   The crosslinking reaction used in the present invention is not limited in any way as long as it allows crosslinkage formation without causing drug denaturation even when crosslinkages are formed in the presence of drugs such as proteins or peptides. Examples of such a reaction

10   include disulfide formation between mercapto groups, addition reaction between a mercapto group and an unsaturated bond, and reaction between a hydrazide group and an activated carboxylic acid ester.

[0024]   The pH during crosslinking is not limited in

15   any way, but it is preferably a pH at which crosslinkage formation is facilitated without causing protein or peptide denaturation so as to prevent reactions with amino groups contained in drugs such as proteins or peptides. Although such a pH can be selected as appropriate by those skilled

20   in the art, it ranges from, for example, pH 3.0 to pH 9.0, and preferably pH 4.5 to pH 9.0.

[0025]   The polysaccharide derivative used in the present invention is not limited in any way as long as it is crosslinkable as described above. Specific examples

25   include hyaluronic acid derivatives (HA derivatives) having a crosslinkable functional group(s) introduced into HA. The crosslinkable functional groups used in the present invention include, without any limitation, a mercapto group,

an unsaturated bond-containing group (e.g., a methacryl group, an acryl group, a vinylsulfone group, an acetylenecarbonyl group), and a hydrazide group (HZ group).

[0026]   In a case where the crosslinking reaction is

5   due to disulfide formation between mercapto groups, for example, crosslinkages can be formed using a polysaccharide derivative such as a HA derivative having mercapto groups, either alone or in combination with a crosslinking agent such as a compound having two or more mercapto groups (e.g.,

10 dithiothreitol (DTT), butanedithiol, polyethylene glycol dithiol, a peptide having two or more cysteines). With the aim of achieving a higher reaction rate of crosslinking, compounds such as sodium tetrathionate (STT), dipyridyl disulfide and Ellman's reagent (DTNB) may be added. In

15 this case, if unreacted mercapto groups remain in the resulting gel, they have a possibility of leading to protein or peptide denaturation. Thus, to maximize the reaction efficiency, these compounds may preferably be added in a 0.1- to 2-fold molar amount, more preferably in

20 a 0.5- to 1.5-fold molar amount, relative to reactive mercapto groups.

[0027]   Although there is no particular limitation on the method for preparing a polysaccharide derivative having mercapto groups, for example, HA may be converted into a

25 tertiary ammonium salt form, dissolved in a polar organic solvent such as DMSO, and then reacted with a mercapto group-containing amine or hydrazide in the presence of a coupling agent. Examples of such a mercapto group-
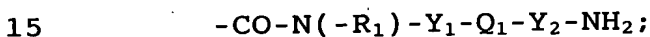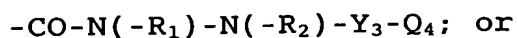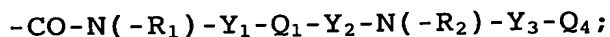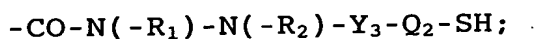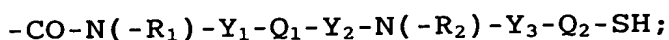
containing amine include, without any limitation, 2-aminoethane-1-thiol, 3-aminopropane-1-thiol, and thioglycolic acid hydrazide.

5   [0028]   In the case of introducing mercapto groups into HA, it is also preferable to first introduce an amino group or a hydrazide group and then introduce a mercapto group into this amino or hydrazide group. For example, carboxylic acid in HA may be condensed with adipic acid dihydrazide (ADH) or a divalent compound containing HZ or

10   amino groups (e.g., ethylenediamine, ethylenedioxybisethylamine) in the presence of a condensing agent to synthesize a hydrazide group-modified HA derivative (HA-HZ) or an amino group-modified HA derivative (HA-amino group), which may then be reacted with, e.g.,

15   N-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) and treated with a reducing agent (e.g., DTT) to form mercapto groups, or alternatively, which may then be reacted with 2-iminothiolane (Trout's Reagent) at the hydrazide or amino groups.

20   [0029]   Examples of a coupling agent include benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), benzotriazol-1-yloxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP), N,N'-carbonyldiimidazole (CDI), N,N'-

25   dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), EDC/3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HODhbt), N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline

(EEDQ), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholium chloride n-hydrate (DMT-MM), and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU).

[0030]   The crosslinkable functional groups intended in the present invention may be introduced, for example, by converting carboxyl groups contained in polysaccharide molecules into mercapto group-, unsaturated bond-, amino group- or hydrazyl group-containing ester or substituted amido groups as shown below:

$-CO-N(-R_1)-Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_2-SH;$

$-CO-N(-R_1)-N(-R_2)-Y_3-Q_2-SH;$

$-CO-N(-R_1)-Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_4;$

$-CO-N(-R_1)-N(-R_2)-Y_3-Q_4;$ or

$-CO-N(-R_1)-Y_1-Q_1-Y_2-NH_2;$

(wherein R_1 represents a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

Y_1 represents a single bond, $-N(-R_3)CO-$, $-N(-R_3)-$, $-CO-$ or $-CH_2CO-$,

Y_2 represents a single bond, $-CON(-R_4)-$ or $-N(-R_4)-$,

Q_1 represents a linear or branched C_{1-10} alkylene group, a linear or branched C_{1-10} hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group,

R_2, R_3 and R_4 each independently represent a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or

branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

Y_3 represents a single bond, -CO-, -CO$_2$-, -CH$_2$-CH(OH)- or -CONH-,

Q_2 represents a linear or branched C_{1-10} alkylene group, a linear or branched C_{1-10} hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group, and
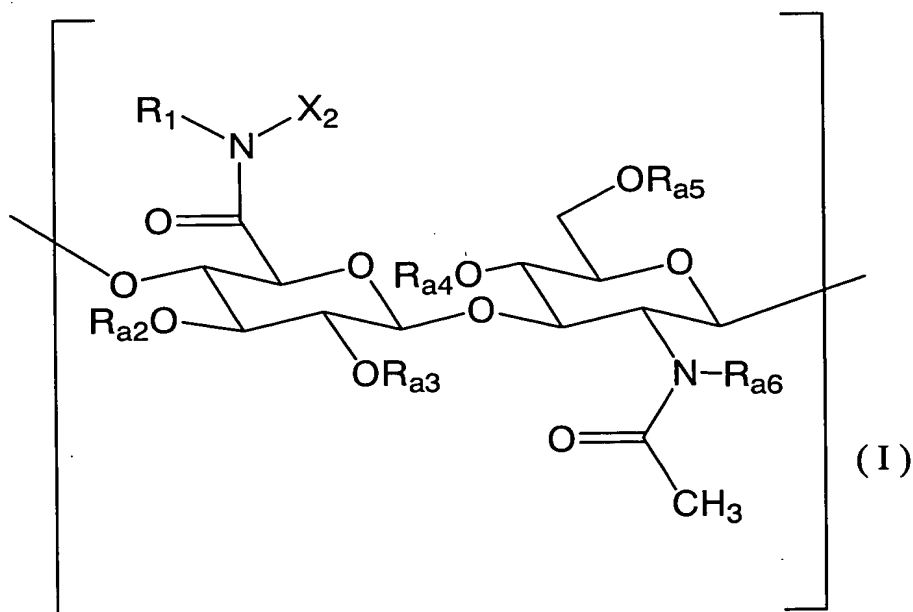
Q_4 represents a linear or branched C_{2-10} alkenyl group or a linear or branched C_{2-10} alkynyl group).

[0031]   Examples of a polysaccharide derivative having mercapto groups preferably include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (I):

[0032] [Formula 1]

(I)

[0033]   (wherein X_2 represents $-Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_2-SH$ or $-N(-R_2)-Y_3-Q_2-SH$,

R_1 represents a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

5   R_{a2}, R_{a3}, R_{a4}, R_{a5} and R_{a6} each independently represent a hydrogen atom, a linear or branched C_{1-6} alkyl group, a linear or branched C_{1-6} alkenyl group, a linear or branched C_{1-6} alkynyl group, a linear or branched C_{1-6} alkylcarbonyl group, a linear or branched C_{1-6} alkenylcarbonyl group, a

10   linear or branched C_{1-6} alkynylcarbonyl group or $-SO_2OH$,

Y_1 represents a single bond, $-N(-R_3)CO-$, $-N(-R_3)-$, $-CO-$ or $-CH_2CO-$,

Y_2 represents a single bond, $-CON(-R_4)-$ or $-N(-R_4)-$,

Q_1 represents a linear or branched C_{1-10} alkylene

15   group, a linear or branched C_{1-10} hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group,

R_2, R_3 and R_4 each independently represent a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or

20   branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

[0034] Y_3 represents a single bond, $-CO-$, $-CO_2-$, $-CH_2-CH(OH)-$ or $-CONH-$, and

Q_2 represents a linear or branched C_{1-10} alkylene group,

25   a linear or branched C_{1-10} hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group).

[0035]   As used in Formula (I), the term "polyalkylene

oxide group" refers to a group represented by $-(CH(-R)CH_2O)_n-H$ (wherein R represents a hydrogen atom or a C_{1-5} alkyl group) and preferably represents a polyethylene oxide group or a polypropylene oxide group. Likewise, n is

5 preferably an integer of 1 to 20. Although the polypeptide group is not limited in any way, it is preferably composed of 1 to 20 amino acids. Likewise, although the polyester group is not limited in any way, it is preferably a polyglycolic acid group or a polylactic acid group.

10 [0036] Moreover, in Formula (I), R_1 preferably represents a hydrogen atom, and X_2 preferably represents $-Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_2-SH$. In Formula (II), Y_1 preferably represents a single bond or $-N(-R_3)-$, Y_2 preferably represents a single bond, and Q_1 preferably represents a

15 linear or branched C_{1-4} alkylene group. In Formula (II), R_2 and R_3 each preferably represent a hydrogen atom, Y_3 preferably represents $-CO-$, and Q_2 preferably represents a linear or branched C_{1-4} alkylene group.

[0037] In a case where addition reaction between a

20 mercapto group and an unsaturated bond is utilized for crosslinking reaction, a polysaccharide derivative such as a HA derivative having unsaturated bond-containing groups may be mixed with a compound having two or more mercapto groups (e.g., dithiothreitol (DTT), butanedithiol,

25 polyethylene glycol dithiol, a peptide having two or more cysteines, a mercapto group-modified HA derivative), or contrariwise, a polysaccharide derivative having mercapto groups may be mixed with a compound having two or more

- 19 -

unsaturated bond-containing groups (e.g., ethylene glycol
dimethacrylate, ethylenebisacrylamide, tris-2-
maleimidoethylamine, 1,8-bismaleimidotriethylene glycol,
1,4-bismaleimidyl-2,3-dihydroxybutane, a HA derivative
5   having unsaturated bonds). In this case, it is preferable
to add a basic compound such as triethanolamine for the
purpose of improving the stability of proteins or peptides
during crosslinking reaction and improving the reaction
rate. The concentration preferred for this purpose is
10   10 µL/mL to 20 µL/mL. Examples of a compound having two or
more mercapto groups also include a linear or branched C_{2-10}
alkylenedithiol (whose alkylene moiety may have one or more
oxygen atoms inserted therein and/or may be substituted
with one or more hydroxyl groups).
15   [0038]   There is no particular limitation on the method
for preparing a polysaccharide derivative having
unsaturated groups, but it is difficult to achieve a high
introduction rate, e.g., when glycidyl ether methacrylate
or methacrylic anhydride is directly reacted with a
20   hydroxyl group in HA (J. Biomed. Mat. Res. 54, 115-121,
2001). This is because HA will form a higher-order
structure in an aqueous solution by the action of hydrogen
bonding or hydrophobic interaction to reduce the reactivity
of its functional groups including hydroxyl and carboxylic
25   acid groups. A higher crosslinking density is desired to
achieve prolonged sustained release of proteins or peptides.
To this end, it is desirable to introduce a substituent at
the carboxyl group of the glucuronic acid moiety. For

example, HA may be converted into a tertiary ammonium salt

form, dissolved in a polar organic solvent such as DMSO,

and then reacted with an unsaturated bond-containing amine

or hydrazide in the presence of a coupling agent such as

5   1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),

benzotriazol-1-yloxy-tris(dimethylamino)phosphonium

hexafluorophosphate (BOP) or benzotriazol-1-yloxy-

trispyrrolidinophosphonium hexafluorophosphate (PyBOP) to

prepare a desired derivative. Examples of such an

10   unsaturated bond-containing amine include, without any

limitation, allylamine, diallylamine, 4-amino-1-butene,

acrylhydrazide, and methacrylhydrazide.

[0039]   It is also preferable to introduce an amino

group or a hydrazide group and then introduce an

15   unsaturated bond-containing group into this amino or

hydrazide group, as described above. For example,

carboxylic acid in HA may be condensed with adipic acid

dihydrazide (ADH) or a divalent compound containing HZ or

amino groups (e.g., ethylenediamine,

20   ethylenedioxybisethylamine) in the presence of a condensing

agent (e.g., EDC, BOP, PyBOP) to synthesize a hydrazide

group-modified HA derivative (HA-HZ) or an amino group-

modified HA derivative (HA-amino group), which may then be

reacted with an unsaturated bond-containing carboxylic acid

25   derivative such as an acid anhydride or activated ester of

R_{10}-COOH (wherein R_{10} represents a linear or branched C_{2-10}

alkenyl group), preferably with methacrylic anhydride,

N-hydroxysuccinimide (NHS)-activated acrylic acid,

methacrylic acid or the like.

[0040]   In a case where polysaccharide (e.g., HA) molecules are crosslinked via mercapto groups after introduction of unsaturated bond-containing groups, the ratio of mercapto groups to unsaturated bond-containing groups is not limited in any way and can be selected as appropriate by those skilled in the art. However, the ratio of mercapto groups to unsaturated bond-containing groups is preferably 3:1 to 1:2, more preferably 2:1 to 1:1, in order to minimize reactions with proteins or peptides, to prevent unsaturated groups from remaining in the gel and to ensure rapid reaction.

[0041]   In a case where HA molecules are crosslinked via unsaturated bond-containing groups after introduction of mercapto groups, the ratio of unsaturated bond-containing groups to mercapto groups is not limited in any way and can be selected as appropriate by those skilled in the art. However, the ratio of unsaturated bond-containing groups to mercapto groups is preferably 3:1 to 1:2, more preferably 2:1 to 1:1, in order to minimize reactions with proteins or peptides, to prevent unsaturated groups from remaining in the gel and to ensure rapid reaction.

[0042]   Examples of a polysaccharide derivative having unsaturated bond-containing groups preferably include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (II):

[0043] [Formula 2]

(II)
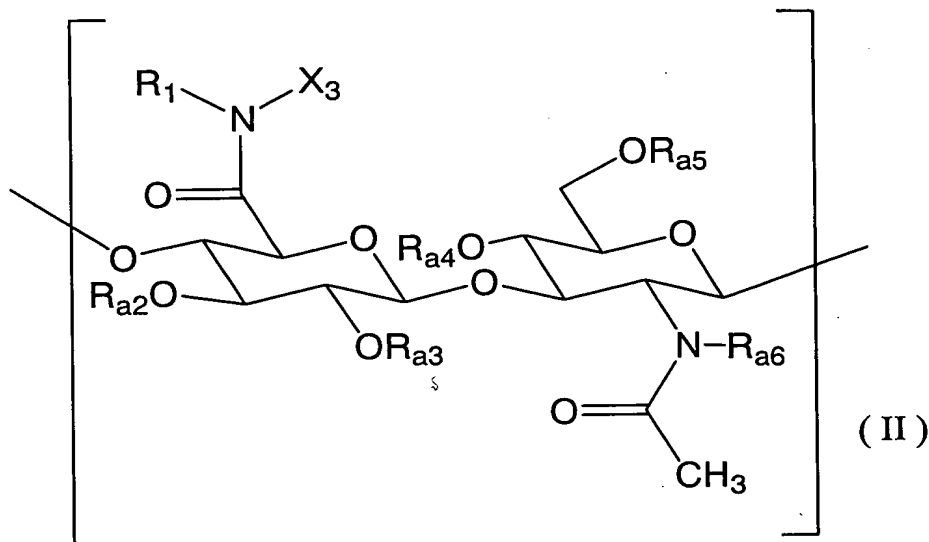
[0044]   (wherein X_3 represents $-Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_4$ or $-N(-R_2)-Y_3-Q_4$,

R_1 represents a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

R_{a2}, R_{a3}, R_{a4}, R_{a5} and R_{a6} each independently represent a hydrogen atom, a linear or branched C_{1-6} alkyl group, a linear or branched C_{1-6} alkenyl group, a linear or branched C_{1-6} alkynyl group, a linear or branched C_{1-6} alkylcarbonyl group, a linear or branched C_{1-6} alkenylcarbonyl group, a linear or branched C_{1-6} alkynylcarbonyl group or $-SO_2OH$,

Y_1 represents a single bond, $-N(-R_3)CO-$, $-N(-R_3)-$, $-CO-$ or $-CH_2CO-$,

Y_2 represents a single bond, $-CON(-R_4)-$ or $-N(-R_4)-$,

Y_3 represents a single bond, $-CO-$ or $-CH_2CO-$,

Q_1 represents a linear or branched C_{1-10} alkylene group, a linear or branched C_{1-10} hydroxyalkylene group, a

polyalkylene oxide group, a polypeptide group or a

polyester group,

R_2, R_3 and R_4 each independently represent a hydrogen

atom, a linear or branched C_{1-10} alkyl group, a linear or

5   branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide

group, a polypeptide group or a polyester group, and

Q_4 represents a linear or branched C_{2-10} alkenyl group

or a linear or branched C_{2-10} alkynyl group).

[0045]   As used in Formula (II), the term "polyalkylene

10   oxide group" refers to a group represented by $-(CH(-$

$R)CH_2O)_n-H$ (wherein R represents a hydrogen atom or a C_{1-5}

alkyl group) and preferably represents a polyethylene oxide

group or a polypropylene oxide group. Likewise, n is

preferably an integer of 1 to 20. Although the polypeptide

15   group is not limited in any way, it is preferably composed

of 1 to 20 amino acids. Likewise, although the polyester

group is not limited in any way, it is preferably a

polyglycolic acid group or a polylactic acid group.

[0046]   Moreover, in Formula (II), R_1 preferably

20   represents a hydrogen atom, and X_3 preferably represents -

$Y_1-Q_1-Y_2-N(-R_2)-Y_3-Q_4$. In Formula (II), Y_1 preferably

represents a single bond, $-N(-R_3)CO-$ or $-N(-R_3)-$, more

preferably $-N(-R_3)CO-$. Likewise, Y_2 preferably represents a

single bond or $-CON(-R_3)-$, more preferably $-CON(-R_3)-$, and

25   Y_3 preferably represents a single bond, $-CO-$ or $-N(-R_3)-$,

more preferably $-CO-$. In Formula (II), Q_1 preferably

represents a linear or branched C_{1-4} alkylene group, R_2 and

R_3 each preferably represent a hydrogen atom, and Q_4

preferably represents a linear or branched C_{2-10} alkenyl group.

[0047]   Likewise, examples of a polysaccharide derivative having mercapto groups include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (I) as mentioned above.

[0048]   Reaction between a polysaccharide derivative such as a HA derivative having hydrazide groups and activated carboxylic acid may also be used for crosslinking reaction. Introduction of hydrazide groups into a polysaccharide may be accomplished in any manner known to those skilled in the art. For example, a carboxyl group in hyaluronic acid may be condensed with a divalent hydrazide-containing compound (i.e., a dihydrazide compound) in the presence of a condensing agent to synthesize a desired derivative. Examples of a dihydrazide compound include succinic acid dihydrazide, glutaric acid dihydrazide, adipic acid dihydrazide, and pimelic acid dihydrazide. Likewise, examples of a condensing agent include 1,3-dicyclohexylcarbodiimide, 1,3-diisopropylcarbodiimide, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. For example, carboxylic acid in hyaluronic acid may be condensed with adipic acid dihydrazide (ADH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to synthesize a hydrazide group-modified hyaluronic acid (HA-HZ). The crosslinking agent is not limited in any way as long as it has a functional group capable of reacting with a HZ group,

and examples include molecules having, in the same molecule,
two or more functional groups such as an NHS-activated
ester group, a pentafluorophenoxycarbonyl group, a p-
nitrophenoxycarbonyl group, an imidazolylcarbonyl group, an
5   isothiocyanato group, a sulfonyl chloride group, a sulfonyl
fluoride group, a formyl group, a vinylsulfonyl group, an
acid anhydride and a 4-nitrophenylformate group.
Illustrative examples of such a crosslinking agent include
bis[sulfosuccinimidyl]suberate, disuccinimidyl glutarate,
10 disuccinimidyl tartrate, and ethylene glycol
bis[succinimidylsuccinate].

[0049]   Taking into account the selective reactivity
with a HZ group relative to an amino group, protein
denaturation and the like, the pH during crosslinking is
15 preferably pH 3.0 to pH 6.0, and more preferably pH 4.0 to
pH 6.0. To maintain the pH within this range during
crosslinking, the buffer to be used is preferably a less
volatile one, as exemplified by citrate buffer. The
crosslinking agent is used such that the amount of its
20 functional groups capable of reacting with a hydrazide
group is preferably 40 mol% or less, more preferably
20 mol% or less, particularly preferably 10 mol% or less,
relative to hydrazide groups in a gel preparation solution.

[0050]   Although there is no particular limitation on
25 the rate of crosslinkable functional groups introduced into
HA, it is preferably 5 mol% or more, particularly
preferably 10 mol% or more, based on glucuronic acid in HA
in order to obtain a non-flowable gel *in vivo*. Moreover,

since the sustained release performance of a drug greatly depends on the crosslinking density of a crosslinked HA derivative, the sustained release period of the drug can be controlled by regulating this introduction rate.

5 [0051]   Examples of a polysaccharide derivative having hydrazide groups include a hyaluronic acid derivative whose molecule contains at least one or more repeated structures represented by Formula (III):

[0052] [Formula 3]

(III)
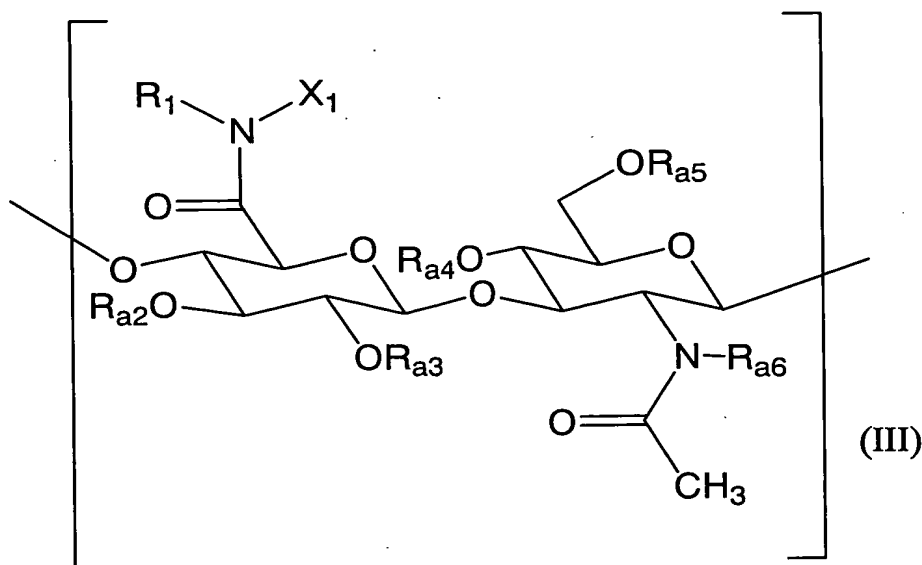
10

[0053]   (wherein R_1 represents a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide group, a polypeptide group or a polyester group,

15   X_1 represents $-Y_1-Q_1-Y_2-NHNH_2$,

R_{a2}, R_{a3}, R_{a4}, R_{a5} and R_{a6} each independently represent a hydrogen atom, a linear or branched C_{1-6} alkyl group, a linear or branched C_{1-6} alkenyl group, a linear or branched

C_{1-6} alkynyl group, a linear or branched C_{1-6} alkylcarbonyl group, a linear or branched C_{1-6} alkenylcarbonyl group, a linear or branched C_{1-6} alkynylcarbonyl group or $-SO_2OH$,

Y_1 represents a single bond, $-N(-R_3)CO-$, $-N(-R_3)-$, -

5 $CO-$ or $-CH_2CO-$,

Q_1 represents a single bond, a linear or branched C_{1-10} alkylene group, a linear or branched C_{1-10} hydroxyalkylene group, a polyalkylene oxide group, a polypeptide group or a polyester group,

10 Y_2 represents a single bond, $-N(-R_4)CO-$, $-CO-$ or -
CH_2CO-, and

R_3 and R_4 each independently represent a hydrogen atom, a linear or branched C_{1-10} alkyl group, a linear or branched C_{1-10} hydroxyalkyl group, a polyalkylene oxide

15 group, a polypeptide group or a polyester group).

[0054]   Moreover, in Formula (III), R_1 preferably represents a hydrogen atom, R_{a2}, R_{a3}, R_{a4}, R_{a5} and R_{a6} each preferably represent a hydrogen atom, Y_1 preferably represents a single bond or $-CO-$, Q_1 preferably represents

20 a linear or branched C_{1-10} alkylene group, Y_2 preferably represents a single bond or $-CO-$, R_3 preferably represents a hydrogen atom, and R_4 preferably represents a hydrogen atom.

[0055]   As used in Formula (III), the term

25 "polyalkylene oxide group" refers to a group represented by $-(CH(-R)CH_2O)_n-OH$ (wherein R represents a hydrogen atom or a linear or branched C_{1-5} alkyl group) and preferably represents a polyethylene oxide group or a polypropylene

oxide group. Likewise, n is preferably an integer of 1 to 20. Although the polypeptide group is not limited in any way, it is preferably composed of 1 to 20 amino acids. Likewise, although the polyester group is not limited in any way, it is preferably a polyglycolic acid group or a polylactic acid group.

[0056] The method of the present invention for preparing crosslinked hyaluronic acid microparticles may be any method in which crosslinking proceeds in parallel with drying of microparticles through solvent distillation. For example, by using a spray dryer for spraying and drying a fluid, a solution containing a drug and a hyaluronic acid derivative having a crosslinkable functional group(s) may be sprayed and dried so as to crosslink the hyaluronic acid derivative during concentration and drying, thereby obtaining drug-carrying microparticles in which the drug is encapsulated in the crosslinked hyaluronic acid. In the case of using the spray-dry technique, the drying temperature is preferably set at 100°C or below in order to prevent drug denaturation.

[0057] Alternatively, a crosslinkable HA derivative (in a tetrabutylammonium salt form) and a drug may be dissolved in a polar organic solvent such as DMSO, followed by addition of a supercritical fluid such as carbon dioxide. DMSO may then be extracted to cause crosslinking reaction during concentration of hyaluronic acid, thereby obtaining microparticles. When using these techniques for microparticle formation, the addition of a surfactant such

as Tween-20 or Tween-80 (around 1-2%) enables improvement
in the recovery rate of the resulting microparticles.
These techniques also require the use of a preparation
solution which does not cause crosslinking reaction before
5   concentration.   The introduction rate of crosslinkable
functional groups is preferably 5 mol% to 70 mol%, the
molecular weight of hyaluronic acid is preferably 10,000 to
2,000,000 daltons, and the concentration of hyaluronic acid
is preferably 0.1% to 5%, although they will vary depending
10   on the time required for a process starting from mixing
with a crosslinking agent until concentration, the
introduction rate of crosslinkable functional groups, as
well as on the molecular weight and concentration of
hyaluronic acid.
15   [0058]   In another method, an aqueous solution
containing a drug and a hyaluronic acid derivative having a
crosslinkable functional group(s) may also be emulsified in
a dehydrating fluid (e.g., polyethylene glycol with a
molecular weight of 400 daltons) to effect crosslinking of
20   hyaluronic acid during dehydration/concentration, thereby
obtaining drug-carrying microparticles in which the drug is
encapsulated in the crosslinked hyaluronic acid. When
using this method, a cationic or nonionic drug is preferred
to improve the encapsulation efficiency.
25   [0059]   Moreover, it is more preferable to perform
thermal treatment after microparticle formation, so that
the water content is further reduced and the crosslinking
reaction is completely terminated. In this case, the

crosslinking density is also improved and a prolonged

sustained release can also be expected. The temperature

required for thermal treatment is not limited in any way,

but it may be performed, for example, at 30°C to 110°C,

5   preferably at 30°C to 60°C.

[0060]   Although the diameter of the dried

microparticles may be optimized for their intended use, the

diameter preferred for injectable purposes is usually

0.01 μm to 150 μm. For transnasal or transpulmonary

10   administration, a diameter of 0.01 μm to 5 μm is preferred

in terms of inhalation efficiency, while a diameter of

around 0.01 μm to 0.2 μm is preferred for intravenous

injection in terms of blood pharmacokinetics.

[0061]   HA used in the present invention may be of any

15   origin, including HA extracted from animal tissues, HA

obtained by fermentation techniques, and HA chemically

synthesized. Moreover, further treatment (e.g.,

hydrolysis) may be performed on HA. Modified HA prepared

in various manners and its salts with alkali metals (e.g.,

20   sodium, potassium, lithium) also fall within the scope of

HA according to the present invention. Although HA is

often modified at its carboxyl or hydroxyl groups, the

modified HA according to the present invention may be

modified at any moiety. Such modified HA is not limited in

25   any way and may receive any modification. Examples include

sulfated HA (WO95/25751), N-sulfated HA (WO98/45335),

esterified HA (EP0216453, WO98/08876, EP0341745),

periodate-oxidized HA, and amide-modified HA.

[0062]   There is no particular limitation on the molecular weight of HA used as a starting material in the present invention, and it is possible to use HA of any molecular weight. In general, HA of 5,000 to 3,500,000 daltons, preferably 10,000 to 1,000,000 daltons, can be used. The molecular weight and concentration of HA will affect the particle diameter of the resulting microparticles and hence may be selected as appropriate for the intended particle diameter.

[0063]   Examples of a pharmacologically active protein or peptide include, without any limitation, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), interferons-α, β and γ (INF-α, β, γ), thrombopoietin (TPO), ciliary neurotrophic factor (CNTF), tumor necrosis factor binding protein (TNFbp), interleukin-10 (IL-10), FMS-like tyrosine kinase (Flt-3), growth hormone (GH), insulin, insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDFG), interleukin-1 receptor antagonist (IL-1ra), brain-derived neurotrophic factor (BDNF), keratinocyte growth factor (KGF), stem cell factor (SCF), megakaryocyte growth and development factor (MGDF), osteoprotegerin (OPG), leptin, parathyroid hormone (PTH), basic fibroblast growth factor (b-FGF), bone morphogenetic protein (BMP), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), glucagon-like peptide-1 (GLP-1), antibody, and diabody. Moreover, the sustained-release drug carrier of the present invention can also be used for drugs of low-molecular-

weight compounds. Examples of such low-molecular drugs
include carcinostatic agents (e.g., alkylating agents,
antimetabolites, alkaloids), immunosuppressive agents,
anti-inflammatory agents (e.g., steroid drugs, non-

5   steroidal anti-inflammatory agents), antirheumatic agents,
and antibacterial agents (e.g., β-lactam antibiotics,
aminoglycoside antibiotics, macrolide antibiotics,
tetracycline antibiotics, novel quinolone antibiotics,
sulfa drugs).

10   [0064]   The sustained-release carrier of the present
invention may be administered as a pharmaceutical
composition in any dosage form suitable for the intended
route of administration, in combination with one or more
pharmaceutically acceptable diluents, wetting agents,

15   emulsifiers, dispersants, auxiliaries, antiseptics, buffers,
binders, stabilizers and the like. The route of
administration may be either parenteral or oral.

BRIEF DESCRIPTION OF DRAWINGS

[0065] [Figure 1] One example of a microscopic photograph

20   showing crosslinked HA-SH microhydrogel microparticles.
[Figure 2] One example of a microscopic photograph showing
crosslinked HA-SH microhydrogel microparticles swollen in
PBS.
[Figure 3] One example of the thermogravimetric results

25   analyzed for an EPO-encapsulated crosslinked HA-SH
microhydrogel.
[Figure 4] A graph showing one example of the RP-HPLC
results, which shows the amount of EPO recovered from the

crosslinked HA-SH microhydrogel microparticles obtained in Examples 1 to 4. Starting from the bottom, the curves correspond to the microparticles obtained in Examples 1, 2, 3 and 4, respectively.

[Figure 5] A graph showing the release profile of EPO from the HA gels obtained in Example 3 and Comparative Example 1.

[Figure 6] One example of the ^{1}H-NMR results measured for the hyaluronic acid derivative (HA-HZ) obtained in Example 9-1.

[Figure 7] One example of the ^{1}H-NMR results measured for the hyaluronic acid derivative (HA-HZ-SH) obtained in Example 9-2.

[Figure 8] One example of the ^{1}H-NMR results measured for the hyaluronic acid derivative (HA-HZ-MA) obtained in Example 10.

[Figure 9] One example of the ^{1}H-NMR results measured for the hyaluronic acid derivative (HA-AM) obtained in Example 11-1.

[Figure 10] One example of the ^{1}H-NMR results measured for the hyaluronic acid derivative (HA-AM-SH) obtained in Example 11-2.

[Figure 11] One example of the ^{1}H-NMR results measured for the hyaluronic acid derivative (HA-AM-MA) obtained in Example 11-1.

[Figure 12] A graph showing curing-induced changes in the water content of the particles obtained in Example 12.

[Figure 13] A graph showing a curing-induced inhibitory effect on swelling of the particles obtained in Example 12.

EXAMPLES

[0066]   Preparation of EPO-encapsulated crosslinked

hyaluronic acid microparticles

The present invention will be further described in

5   more detail in the following preferred examples, which are

not intended to limit the scope of the invention.

[0067]   NMR measurement was carried out using a nuclear

magnetic resonance system JNM-ECA500 (JEOL. Ltd., Japan)

and heavy water (D_2O) as a solvent. The introduction rate

10   of each substituent was determined from the integral ratio

between a peak unique to the introduced substituent and a

peak derived from hyaluronic acid.

[Example 1]

[Example 1-1] Synthesis of hydrazide (HZ) group-modified

15   hyaluronic acid derivative (HA-HZ)

[0068] [Formula 4]

HA

HA-HZ
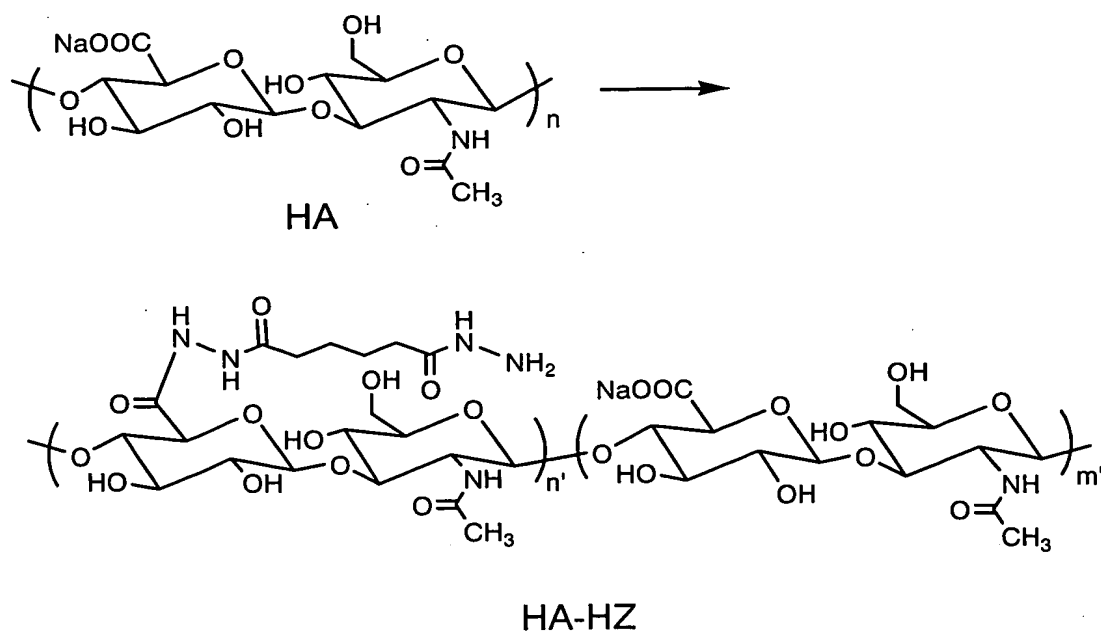
[0069]   Hyaluronic acid (HA) having a molecular weight

of 1.9×10^5 daltons (200 mg, Denki Kagaku Kogyo Kabushiki

Kaisha, Japan) was dissolved in distilled water at a

concentration of 0.5% and adjusted with 5N hydrochloric

acid to pH 4.7 to 4.8. 1-Ethyl-3-(3-

5 dimethylaminopropyl)carbodiimide (EDC) and adipic acid

dihydrazide (ADH) were added at a molar ratio of HA:EDC:ADH

= 1:0.3:40 (Batch 1-1), 1:1:40 (Batch 1-2) or 1:5:40 (Batch

1-3), and reacted while stirring at room temperature for

2 hours and adjusting the mixture with 5N hydrochloric acid

10 to maintain a pH of 4.7 to 4.8. The reaction mixture was

dialyzed against a 100 mM sodium chloride solution and a

25% ethanol solution (SpectraPor 7, molecular weight cutoff

(MWCO): 12k-14k daltons) and lyophilized to give the titled

HA-HZ.

15 [0070]   The introduction rate of HZ groups in the

resulting HA-HZ was determined for each batch by proton NMR,

indicating that carboxylic acid in HA was modified with HZ

at a rate of 26% (Batch 1-1), 46% (Batch 1-2) or 69% (Batch

1-3) (calculated by comparing N-acetyl groups in HA and

20 HA-HZ (1.9 ppm, 3H) and methylene groups in the adipic

acid-derived moiety of HA-HZ (1.6 ppm, 2.3 ppm, 2H each)).

[Example 1-2] Synthesis of mercapto (SH) group-modified

hyaluronic acid derivative (HA-SH)

[0071] [Formula 5]

HA-HZ ⟶

HA-HZ-SH
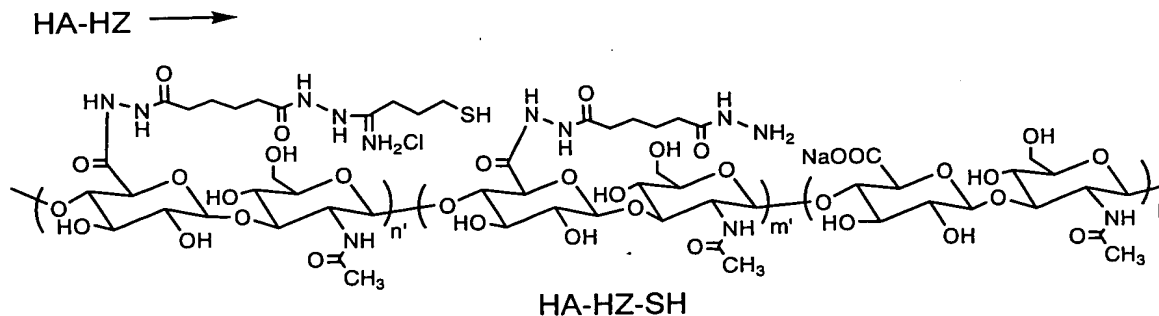
[0072]   HA-HZ obtained in Batches 1 to 3 of Example 1-1
(100 mg each) was dissolved in 5 mL of 100 mM phosphate
buffer, pH 8 (HA-HZ: 2% w/v), followed by addition of
5   iminothiolane (ITL) (at a molar ratio of HZ/ITL = 1/2).
The mixture was reacted while stirring at room temperature
for 2 to 4 hours and then precipitated in ethanol, washed
three times and dried. The introduction rate of SH groups
in the resulting HA-SH was determined for each batch by
10   proton NMR and the results obtained are shown in Table 1
(calculated by comparing N-acetyl groups in HA and HA-SH
(1.9 ppm, 3H) and methylene groups in the ITL-derived
moiety of HA-SH (2.1 ppm and 2.7 ppm, 2H each)).

[0073] [Table 1]

15   Table 1

	Control	Batch 1	Batch 2	Batch 3
HZ introduction	0%	26%	46%	69%
SH introduction	0%	20%	35%	56%

[0074] [Example 1-3] Preparation of EPO-encapsulated
crosslinked hyaluronic acid microparticles

     HA-SH (introduction rate of SH groups: 20 mol%)
20   obtained in Batch 1 of Example 1-2 (200 mg) and

erythropoietin (EPO; 2 mg) were dissolved in 20 mL of 10 mM

phosphate buffer (PB; pH 8) (stirring at room temperature

for 1 hour), followed by addition of Tween-20 (4 mg) and

sodium tetrathionate (STT; 22.3 mg, 1-fold molar amount

5   relative to SH groups). This solution was spray-dried

under the following conditions to obtain microparticles.

Spray dryer: Buchi Mini spray dryer B-191

Solution feed rate: 1.5 mL/min (Tygon tube, Pump speed =

15%)

10 Feed solution concentration: 10 mg/mL

Atomizing air flow rate: 650 L/hr

Drying air flow rate: 40 kL/hr (Aspiration speed = 65%)

Inlet temperature: 85°C to 95°C

Outlet temperature: 50°C to 60°C

15 [Example 2]

The same procedure as shown in Example 1-3 was

repeated to prepare EPO-encapsulated crosslinked hyaluronic

acid microparticles, except that HA-SH (introduction rate

of SH groups: 35 mol%) obtained in Batch 2 (200 mg) and

20 sodium tetrathionate (STT; 39.0 mg, 1-fold molar amount

relative to SH groups) were used in the experimental

operation of Example 1-3.

[Example 3]

The same procedure as shown in Example 1-3 was

25 repeated to prepare EPO-encapsulated crosslinked hyaluronic

acid microparticles, except that HA-SH (introduction rate

of SH groups: 56 mol%) obtained in Batch 3 (200 mg) and

sodium tetrathionate (STT; 62.4 mg, 1-fold molar amount

relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 4]

The same procedure as shown in Example 1-3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that HA-SH (introduction rate of SH groups: 56 mol%) obtained in Batch 3 (200 mg) and sodium tetrathionate (STT; 38.9 mg, 0.7-fold molar amount relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 5]

The same procedure as shown in Example 1-3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that HA-SH (introduction rate of SH groups: 56 mol%) obtained in Batch 3 (200 mg) and sodium tetrathionate (STT; 27.8 mg, 0.5-fold molar amount relative to SH groups) were used in the experimental operation of Example 1-3.

[Example 6]

The same procedure as shown in Example 3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that Tween-20 (4 mg) was replaced by Tween-80 (4 mg) in the experimental operation of Example 3.

[Example 7]

The same procedure as shown in Example 3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that Tween-20 was not added in the experimental operation of Example 3.

[Example 8]

The same procedure as shown in Example 3 was repeated to prepare EPO-encapsulated crosslinked hyaluronic acid microparticles, except that STT was not added in the

5 experimental operation of Example 3.

[Comparative Example 1]

HA-SH (introduction rate of SH groups: 56 mol%) prepared in Batch 3 of Example 1-2 (33 mg) was dissolved in 690 µL of 10 mM phosphate buffer (pH 8.0), supplemented

10 with 30 µL of an aqueous EPO solution (10 mg/mL) and stirred for 10 minutes, followed by addition of a solution prepared by dissolving sodium tetrathionate (STT; 9.3 mg, 1-fold molar amount relative to SH groups) in 30 µL of 10 mM phosphate buffer, pH 8.0. The resulting solution was

15 filled in 250 µL volumes into 1 mL syringes and reacted at 37°C for 5 hours to obtain cylindrical HA gels.

[Comparative Example 2]

The same procedure as shown in Example 8 was repeated to prepare EPO-encapsulated HA microparticles, except that

20 HA-SH was replaced by HA in Example 8.

[0075]   It should be noted that the microparticles obtained in Examples 1 to 8 and Comparative Example 2 were each collected at a rate of 50% to 65%.

[Test Example 1] Measurement of particle diameter and

25 particle water content

Figure 1 shows a microscopic photograph of the microparticles prepared in Example 3 (×3000 magnification). Figure 2 shows a microscopic photograph of these

microparticles when dispersed in PBS (×3000 magnification). The particle diameter of these microparticles was about 1.2 μm (dried) and about 1.8 μm (swollen with water).

[0076]   Thermogravimetry analysis (TGA) was carried out to determine the water content of the microparticles prepared in Example 3 (Figure 3). The water content was about 15%.

[Test Example 2] Measurement of EPO recovery rate in EPO-encapsulated crosslinked hyaluronic acid microparticles

The microparticles prepared in Examples 1 to 8 and Comparative Example 2 (5 mg each) were each dispersed in 0.5 mL PBS, supplemented with 0.25 units of Hyaluronidase SD (HAse; Seikagaku Corporation, Japan) and enzymatically treated at 25°C for 3 hours to completely digest the microparticles. Likewise, the gel prepared in Comparative Example 1 (0.25 mL) was supplemented with 0.75 mL PBS (pH 7.4) containing 0.5 units of Hyaluronidase SD (Seikagaku Corporation, Japan) and enzymatically treated at 25°C for 1 day to completely digest the gel. The resulting solutions after enzymatic treatment (0.15 mL each) were used as sample solutions. These sample solutions were measured by reversed-phase chromatography (RP-HPLC) using a 0.1 mg/mL aqueous EPO solution as a standard solution to calculate the EPO concentration in each sample solution from the peak area ratio between standard solution and sample solution. The recovery rate was calculated as the amount of EPO determined by RP-HPLC relative to the amount of EPO added (0.1 mg/gel).

[0077]   High performance liquid chromatography analysis on a reversed-phase column (RP-HPLC) was carried out using a Waters 600S controller, a 717 plus autosampler and a 486 infrared absorption spectrometer (Waters) under the

5   following measurement conditions.

Column: C4 (particle diameter: 5 μm, size: 4.6 × 250 mm)

Mobile phases:

   A: water/acetonitrile/trifluoroacetic acid = 400/100/1

   B: water/acetonitrile/trifluoroacetic acid = 100/400/1

10   Flow rate: 1 mL/minute, eluted with a gradient of the mobile phases A/B = 65/35 to 0/100

Column temperature: around room temperature

Sample temperature: 4°C

Detection wavelength: UV 280 nm

15   Analysis software: Millenium 32 ver. 3.21

   When measured as described above, the recovery rate of EPO relative to the initial amount was as follows.

Examples 1 to 4: 90% to 95%

Examples 5 and 6: 80% to 85%

20   Examples 7 and 8: 75% to 80%

Comparative Examples 1 and 2: 90% to 95%

   These results confirmed that the recovery rate was improved by addition of STT and/or a surfactant.

[Test Example 3] Sustained release of EPO from the prepared

25   EPO-encapsulated HA hydrogels

   The microhydrogel prepared in Example 3 (20 mg) and the bulk gel prepared in Comparative Example 1 (250 μL) were each incubated in 2 mL PBS at 37°C and sampled over

time in 200 μL aliquots. The amount of EPO released into the buffer was determined by RP-HPLC.

[0078]   Figure 1 shows the release profile of EPO from each gel, as determined by assuming that EPO collected from the gel digested with hyaluronidase immediately after gel preparation is set to 100%. After 9 days, hyaluronidase (HAse) was added.

[0079]   The results indicate that EPO in the gels remains undenatured and is rapidly released from the gel obtained in Comparative Example 1 because of its low crosslinking density. The results also indicate that the microgel of Example 3 allows sustained release of about 30% EPO over about 5 days because of its high crosslinking density, while 40% of EPO is not released by diffusion but can be released upon enzymatic digestion.

[0080]   By using drug-carrying microparticles, in which a drug is encapsulated in crosslinked hyaluronic acid, as illustrated in the above examples, it is possible to prepare injectable sustained-release formulations which allow long-term release of drugs such as proteins or peptides crosslinked *in situ*, dried and encapsulated in gel microparticles while retaining their biological activity.

[Example 9]

[Example 9-1] Synthesis of hydrazide (HZ) group-modified hyaluronic acid derivative HA-HZ (mixed solvent technique)

[0081] [Formula 6]

HA

HA-HZ
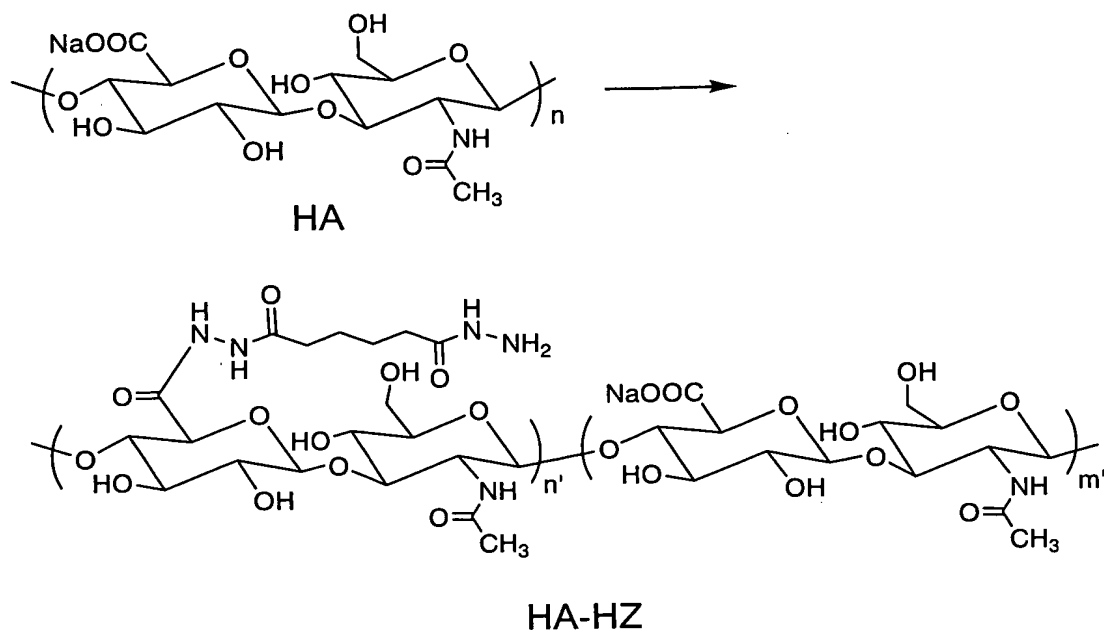
[0082]   HA having a molecular weight of 2×10^5 daltons
(76.0 mg, Denki Kagaku Kogyo Kabushiki Kaisha, Japan) was
5   dissolved at a concentration of 0.1% in distilled
water/EtOH = 50/50 and adjusted with 5N hydrochloric acid
to pH 4.7 to 4.8. 1-Ethyl-3-(3-dimethylaminopropyl)-
carbodiimide (EDC) and adipic acid dihydrazide (ADH) were
added at a molar ratio of HA unit (1 unit = repeated unit
10 of N-acetylglucosamine-glucuronic acid):EDC:ADH = 1:4:40,
and reacted at room temperature for 2 hours while adjusting
the mixture with 5N hydrochloric acid to maintain a pH of
4.7 to 4.8. The reaction mixture was dialyzed sequentially
against large excess volumes of a 100 mM sodium chloride
15 solution, a 25% ethanol solution and distilled water
(SpectraPor 7, molecular weight cutoff (MWCO): 12k-14k
daltons) and lyophilized to give 57.0 mg of the titled
hydrazide (HZ) group-modified hyaluronic acid (HA-HZ). The

introduction rate of HZ groups in the resulting HA-HZ was
determined as the introduction rate of ADH by proton NMR
(calculated by comparing N-acetyl groups (1.85 ppm) for HA
and ADH-derived 4 methylene groups (1.5, 2.1 and 2.25 ppm)

5   for HZ). The introduction rate of HZ was 47%.

[Example 9-2] Synthesis of mercapto (SH) group-modified
hyaluronic acid derivative HA-HZ-SH

[0083] [Formula 7]
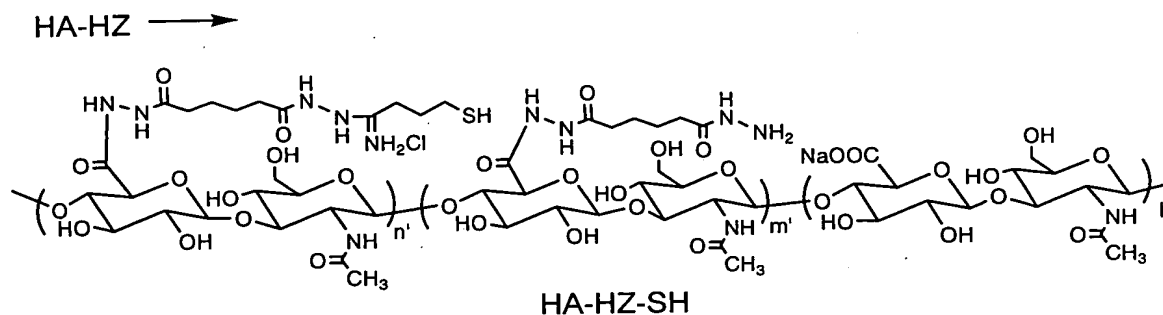
HA-HZ-SH

10

[0084]   HA-HZ synthesized in the same manner as used in
Example 9-1 (100 mg) was dissolved in 5 mL of 100 mM
phosphate buffer, pH 8 (HA-HZ: 2% w/v), followed by
addition of iminothiolane (ITL) (at a molar ratio of HZ/ITL

15 = 1/2). The mixture was reacted while stirring at room
temperature for 2 hours and then precipitated in ethanol,
washed three times and dried. The introduction rate of SH
groups in the resulting HA-HZ-SH was determined by proton
NMR, indicating that the introduction rate of SH was 37.5

20 mol% (calculated by comparing N-acetyl groups in HA and
HA-HZ-SH (1.9 ppm, 3H) and methylene groups in the ITL-
derived moiety of HA-HZ-SH (2.1 ppm and 2.7 ppm, 2H each)).

[Example 10] Synthesis of methacryloyl (MA) group-modified

hyaluronic acid derivative HA-HZ-MA

[0085] [Formula 8]

HA-HZ →

HA-HZ-MA
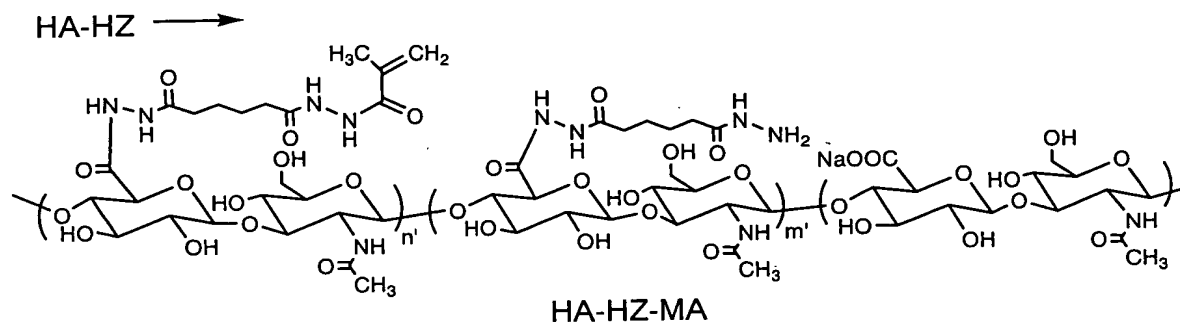
[0086]   The same procedure as used in Batch 3 of

Example 1-1 was repeated to synthesize HA-HZ (63% of

carboxylic acid in HA was modified with HZ), except that

the molecular weight of HA was set to 2×10^4 daltons. The

resulting HA-HZ was dissolved in distilled water, followed

by addition of 1M phosphate buffer (pH 8.8) to prepare

0.1 M phosphate buffer having a HA concentration of

50 mg/mL. Methacrylic anhydride was added dropwise in an

amount of 20-fold equivalents relative to HZ and reacted

while stirring overnight at room temperature. After

precipitation in tetrahydrofuran, the precipitate was

collected and dried. This precipitate was dissolved in

distilled water, precipitated again in tetrahydrofuran and

then dried. The dried product was then dissolved in

distilled water and lyophilized to give the titled HA-HZ-MA.

[0087]   The introduction rate of methacryloyl groups

was determined by proton NMR (calculated by comparing

methyl protons in N-acetyl groups (1.8 to 1.9 ppm) for HA

and $CH_2=$ in methacryloyl groups (5.5 to 6.1 ppm) for MA).

The introduction rate of MA was 22%.

[Example 11]

[Example 11-1] Synthesis of amino (AM) group-modified hyaluronic acid derivative HA-AM

[0088] [Formula 9]

HA-TBA

HA-AM
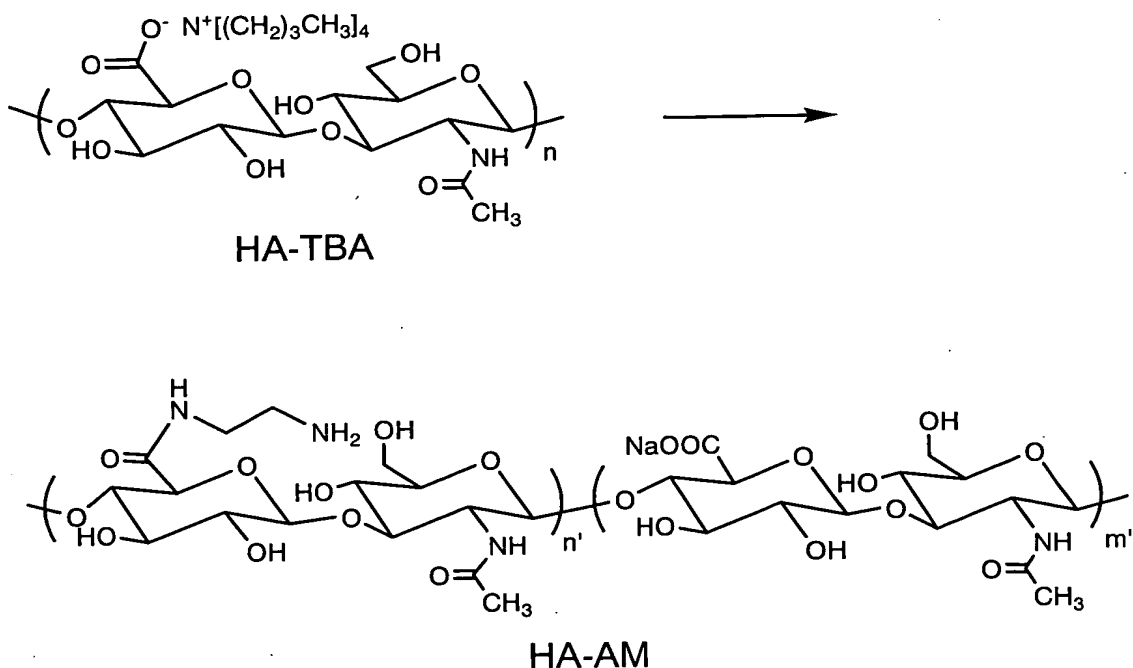
5

[0089]   Sodium hyaluronate (HA) having a molecular weight of 2.0 × 10^5 daltons (Denki Kagaku Kogyo Kabushiki Kaisha, Japan) was converted into a tetrabutylammonium

10   (TBA) salt form using DOWEX 50WX8-400 (Sigma-Aldrich Corporation) which had been treated with tetrabutylammonium hydroxide (Sigma-Aldrich Corporation).

[0090]   After the resulting tetrabutylammonium salt of hyaluronate (HA-TBA) was dissolved at a concentration of

15   2.0 mg/mL in DMSO (Wako Pure Chemical Industries, Ltd., Japan), ethylenediamine (EDA; Sigma-Aldrich Corporation) and BOP (Wako Pure Chemical Industries, Ltd., Japan) were

added in this order at an equivalent ratio of HA

unit/BOP/EDA = 1/2.5/50 (mol/mol/mol) and reacted overnight

at room temperature. After 1M aqueous sodium chloride was

added in an amount of half the volume of the reaction

5   solution, the resulting mixture was reduced to pH 3 with 5N

HCl and further neutralized with 2N NaOH. The reaction

mixture was dialyzed sequentially against large excess

volumes of 0.3 M aqueous sodium chloride and distilled

water (SpectraPor 4, molecular weight cutoff (MWCO):

10 12k-14k daltons), ultrafiltered and lyophilized to give the

titled amino group-modified hyaluronic acid (HA-AM).

[0091]   The introduction rate of amino groups was

determined by proton NMR (calculated by comparing methyl

protons in N-acetyl groups (1.8 to 1.9 ppm) for HA and

15 methylene protons in the ethylenediamine moiety (2.9 to

3.1 ppm) for AM). The introduction rate was 88.5%.

[Example 11-2] Synthesis of mercapto (SH) group-modified

hyaluronic acid derivative HA-AM-SH

[0092] [Formula 10]

HA-AM ⟶

HA-AM-SH
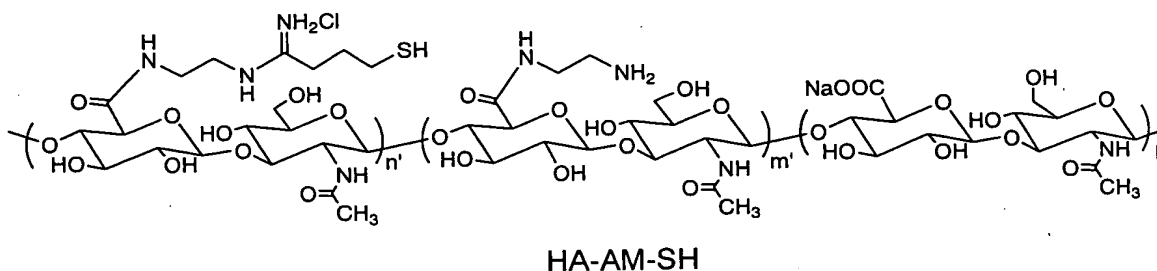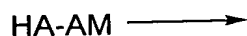
20

[0093]   After HA-AM obtained above was dissolved at

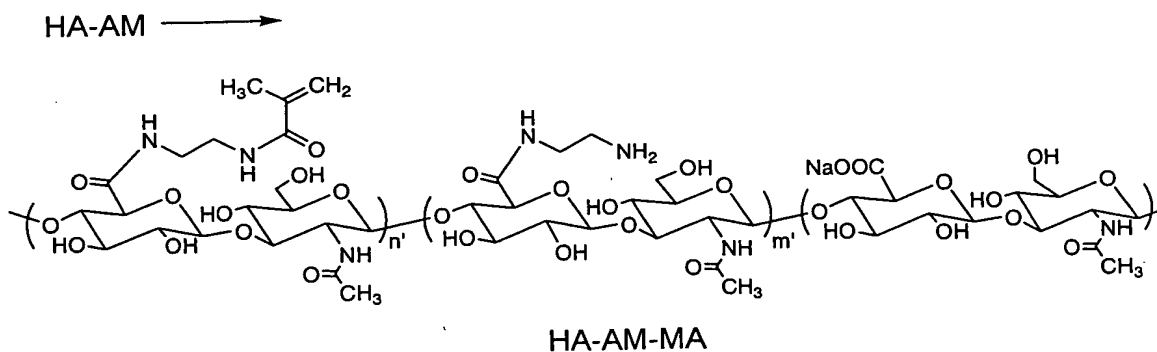2 mg/mL in carbonate buffer (pH 9), iminothiolane (Pierce)

was added in an amount of 0.5- or 1-fold equivalent relative to HA units and reacted for 45 minutes at room temperature. After the reaction, the reaction mixture was purified on a PD-10 column (Amersham Biosciences)

5   equilibrated with 0.005 N aqueous HCl, and then lyophilized to remove the solvent. The resulting polymer was washed with an excess volume of ethanol and dried under reduced pressure to give HA-AM-SH.

[0094]   The introduction rate of mercapto groups was

10   determined by proton NMR in a state containing a reducing agent tris(2-carboxyethylphosphine) hydrochloride (TCEP) (calculated by comparing methyl protons in N-acetyl groups (1.8 to 1.9 ppm) for HA and methylene protons adjacent to mercapto groups (2.4 to 2.7 ppm) for SH). The introduction

15   rate was 16.5% and 23.5% in the respective cases.

[Example 11-3] Synthesis of methacryloyl (MA) group-modified hyaluronic acid derivative HA-AM-MA

[0095] [Formula 11]

HA-AM ⟶

HA-AM-MA

20

[0096]   After HA-AM obtained above was dissolved at 10 mg/mL in phosphate buffer (pH 7), methacrylic acid activated with 1-ethyl-3-(3-

dimethylaminopropyl)carbodiimide (EDC) was added in an amount of 0.5-, 1.0- or 2.0-fold equivalents relative to HA units and reacted for 2 hours at room temperature. After the reaction, the reaction mixture was dialyzed sequentially against 0.3 M aqueous sodium chloride and distilled water (SpectraPor 4, molecular weight cutoff (MWCO): 12k-14k daltons) for purification purposes, and then lyophilized to give the above polymer.

[0097]   The introduction rate of methacryloyl groups was determined by proton NMR (calculated by comparing methyl protons in N-acetyl groups (1.8 to 1.9 ppm) for HA and $CH_2=$ in methacryloyl groups (5.5 to 6.1 ppm) for MA). The introduction rate was 14.8%, 31.9% and 68.9% in the respective cases.

[Example 12] Effect of thermal treatment on microparticles (swelling inhibition)

HA-HZ-SH (introduction rate of SH groups: 37.5 mol%) synthesized in Example 9-2 (100 mg) was dissolved in 8.5 mL of distilled water. To this solution, 1 mL of 100 mM phosphate buffer (PB; pH 7) was added and 5 mg of Tween-80 was further dissolved, followed by addition of STT (2.3 mg, 1/10-fold molar amount relative to SH groups). This solution was spray-dried under the same conditions as used in Example 1-3 (Solution feed rate 0.5 mL/min, Aspiration speed = 100%) to obtain microparticles. These microparticles were cured in a 50°C thermostat (DN-42, Yamato Scientific Co., Ltd., Japan) and sampled after 24 and 72 hours.

[Test Example 4]

Each sample sampled in Example 12 was measured for particle water content by TGA. The results obtained are shown in Figure 12. Likewise, 30 particles were randomly selected from each sample and measured for their Feret's diameter (dry particle diameter) by microscopic image analysis. Further, the sampled particles were swollen by addition of PBS containing Tween-80 (0.05%) and measured for their wet particle diameter in the same manner. The results obtained are shown in Figure 13. These results confirmed that the degree of swelling was reduced when the particles were incubated for 24 hours. This would be because incubation at 50°C for 24 hours may increase crosslinking within the particles.

[Example 13] Preparation of crosslinked HA-HZ-MA microgels

HA-HZ-MA synthesized in Example 10 (100 mg) was dissolved in 6 mL of distilled water. To this solution, 1 mL of 100 mM phosphate buffer (PB; pH 8.5) containing DTT (11 mg) and TEA (32.5 µL) was added and further mixed with distilled water (3 mL). This solution was spray-dried under the same conditions as used in Example 12 (exhaust temperature 65°C) to obtain microparticles. These microparticles were cured in a 50°C thermostat (DN-42, Yamato Scientific Co., Ltd., Japan) for about 72 hours to obtain particles.

[Test Example 5]

The particles obtained in Example 13 were placed on a slide and, after addition of PBS (pH 7), were then observed

for their state under a microscope, indicating that the particles were not dissolved in PBS. This microscopic observation confirmed crosslinkage formation caused by addition reaction between a mercapto group and an

5 unsaturated bond in the microparticles obtained in Example 13.

INDUSTRIAL APPLICABILITY

[0098]   The sustained-release drug carrier of the present invention allows *in situ* chemical crosslinking of

10 drugs such as proteins or peptides and their encapsulation into HA gels while retaining their biological activity, and enables the provision of injectable microparticles which allow long-term sustained release of the drugs such as proteins or peptides at high recovery rates.

15